



BEST AVAILABLE COPY

Journal of Biotechnology 34 (1994) 269–287

---

 journal of  
**biotechnology**


---

## Minireview

## Production of pharmaceutical proteins from transgenic animals

Louis-Marie Houdebine

*Unité de Différenciation Cellulaire, Institut National de la Recherche Agronomique, 78352 Jouy-en-Josas Cédex, France*

(Received 4 October 1993; revision accepted 21 January 1994)

**Abstract**

Different systems are being studied and used to prepare recombinant proteins for pharmaceutical use. The blood, and still more the milk, from transgenic animals appear a very attractive source of pharmaceuticals. The cells from these animals are expected to produce well-matured proteins in potentially huge amounts. Several problems remain before this process becomes used in a large scale. Gene transfer remains a difficult and costly task for farm animals. The vectors carrying the genes coding for the proteins of interest are of unpredictable efficiency. Improvement of these vectors includes the choice of efficient promoters, introns and transcription terminators, the addition of matrix attached regions (MAR) and specialized chromatin sequences (SCS) to enhance the expression of the transgenes and to insulate them from the chromatin environment. Mice are routinely used to evaluate the gene constructs to be transferred into larger animals. Mice can also be utilized to prepare amounts as high as a few hundred mg of recombinant proteins from their milk. Rabbit appears adequate for amounts not higher than 1 kg per year. For larger quantities, goat, sheep, pig and cow are required.

No recombinant proteins extracted from the blood or milk of transgenic animals are yet on the market. The relatively slow but real progress to improving the efficiency of this process inclines to be reasonably optimistic. Predictive reports suggest that 10% of the recombinant proteins, corresponding to a 100 million dollars annual market, will be prepared from the milk of transgenic animals by the end of the century.

**Key words:** Pharmaceutical protein; Transgenic animal; Milk

**1. Introduction**

The preparation of recombinant proteins was one of the first successes of biotechnology, and more and more of these molecules are obtained and used each year. Indeed, recombinant proteins, including antibodies, are being used as pharmaceuticals for human and animal therapy (hormones, growth factors, antibodies), as antigens for vaccinations, as reagents in biochemical diagnosis, as enzymes in chemical industry, and as an experimental material in many research

laboratories. Preparing recombinant proteins rather than native proteins from living organisms offers several advantages. In some cases, for example, erythropoietin, the protein is so rare in nature that the recombinant molecule is the only possible source for human therapy. In other cases, for example, insulin or human growth hormone, the recombinant is preferred to its counterpart from pig or human origin for safety reasons. More generally, genetic engineering most of the time offers the best way to obtain a given protein (and its mutants) in large amounts.

Table 1  
Some of the major post-transcriptional modifications of proteins

|   |
|---|
| ♦ Maturation of precursors (proteolysis)                |
| ♦ Cleavage of signal peptide                            |
| ♦ Withdrawal of the methionine from NH <sub>2</sub> end |
| ♦ Formation of S-S bridges                              |
| ♦ Folding   |
| ♦ Glycosylation (O <sup>-</sup> and N <sup>-</sup> )    |
| ♦ Amidation   |
| ♦ Myristoylation  |
| ♦ Acetylation   |
| ♦ Phosphorylation                                       |
| ♦ Phenylation   |
| ♦ Association of subunits                               |

The genetic code being universal, any gene from any origin can be theoretically expressed in the corresponding protein in any cell type as soon as the appropriate regulatory region has been associated with the coding part of the gene. The reality is in practice more complex. The vectors containing the gene to be expressed are in a certain number of cases of a poor efficiency, in an unpredictable manner. The recombinant proteins are sometimes not easily extractable from the reprogrammed cells. Moreover, a protein is not only a linear chain of amino acids. To be biologically active and stable *in vivo*, many proteins have to be properly folded and modified post-transcriptionally. A list of these modifications is summarized in Table 1. The cells reprogrammed with the foreign genes show variable capacity to synthesize, to mature and to secrete the corresponding recombinant proteins which they synthesize. For these reasons, several biological systems are being used and studied with the hope to improve them and, thus, to prepare large amounts of recombinant proteins at lower cost.

## 2. Why use transgenic animals?

The different systems which are being used to prepare recombinant proteins are listed in Table 2. Bacteria are easily reprogrammed, usually they synthesize the foreign proteins very efficiently, but are unable to perform some of the post-translational modifications, and particularly glycosylation, which takes place in the Golgi apparatus of

eukaryotic cells. Most of the bacteria used until now do not secrete the recombinant proteins which are sometimes very difficult to extract (Blum et al., 1992). Theoretically, recombinant bacteria can synthesize as much pharmaceutical protein as desired.

Yeast is also easily reprogrammed and it can secrete proteins which are glycosylated. The enzymatic machinery which glycosylates proteins is, however, different in lower and higher eukaryotes, and yeast often secretes recombinant molecules inadequately glycosylated (Buckholz and Gleeson, 1991). As bacteria, yeast can theoretically be cultured in batches as numerous as wanted.

Fungi offer interesting possibilities for relatively large-scale preparations of recombinant proteins. The proteins prepared in this manner are not expected to have received appropriate post-translational modifications in all cases.

Transgenic plants can be obtained easily, at least for some species, and cultured in very large areas. They may be an ideal 'living fermentor' to prepare very large quantities of non-glycosylated proteins, such as human albumin (Sijmons et al., 1990).

In culture, animal cells can be easily reprogrammed using conventional transfection and infection with viral vectors. A certain number of cell lines can be maintained in fermentors and be the source of recombinant proteins. The post-translational modifications of the recombinant proteins prepared in this manner are usually quite similar to those observed in the native proteins. A

Table 2  
The different systems producing recombinant proteins

|                    | Amount | Extraction | Post-translational modifications |
|--------------------|--------|------------|----------------------------------|
| Bacteria           | ++++   | ++         | +                                |
| Yeast              | ++++   | +++        | ++                               |
| Fungi              | ++++   | +++        | ++                               |
| Transgenic plants  | ++++   | ++?        | ++                               |
| Baculovirus        | ++++   | +++        | +++                              |
| Mammalian cells    | +      | ++++       | ++++                             |
| Transgenic animals | ++++   | ++++       | ++++                             |

Each of these systems has advantages and drawbacks roughly estimated by the cross in the table

relatively large variety of cell lines are already available for this purpose and their number can be increased after an immortalization of primary cells with oncogenes. Animal cells in culture thus offer virtually all the possibilities to synthesize bona fide recombinant proteins. The only limitation comes from the fact that animal cell culture remains relatively costly and cannot be easily developed on a very large scale as compared to bacteria or yeast. Hence, only recombinant proteins to be used in relatively small quantities, such as human erythropoietin, factor VIII or tissue plasminogen activator, are prepared from cultured animal cells (Datar et al., 1993).

Transgenic animals share most of the properties of animal cells in culture. They can properly carry out the post-translational modifications of recombinant protein. Cells in the animal are in an ideal metabolic situation and they can synthesize and secrete proteins very efficiently. These cells are as numerous as wanted as soon as transgenic animals are multiplied. Transgenic animals are, therefore, theoretically ideal fermentors. However, many problems remain to be solved before they can be used on a large scale.

### 3. Which transgenic animals to use

The first idea which comes to mind is to extract the recombinant proteins from the biological

fluids of the transgenic animals (Table 3). In this respect, blood appears to be a good candidate. It is abundant and a low cost by-product of slaughterhouses. Many of the proteins secreted by the cells are found in the blood of animals. Hence, vectors driving the expression of the foreign genes in organs, such as liver or in most cell types of the animal, can be used. Such vectors are available. However, most of the blood proteins, at least those to be used for therapeutical purposes, are unstable and rapidly cleared from the circulation. These proteins have little chance to accumulate in blood in sufficient quantities to be used as the source of pharmaceuticals. This is the case for human growth hormone which has a half-life of about 15 min and which is, thus, not found at concentrations higher than a few  $\mu\text{g ml}^{-1}$  in the blood of most transgenic mice, although their transgene is expressed at a high rate in many tissues under the control of strong promoters such as that of metallothionein gene (Palmiter et al., 1983). On the other hand, the recombinant proteins which are presently being prepared are essentially those having pharmaceutical properties in humans. Animals used for transgenesis, essentially mammals, are evolutionarily close to humans. The human recombinant proteins present in blood at high concentrations are expected to be active in the transgenic animals in most cases, and to alter more and less heavily the

Table 3  
The different transgenic animals and biological fluids which can be used for the preparation of recombinant proteins

| Source            | Amount | Purification | Potential toxicity | Transgenesis | Post-translational modifications | Specific advantages and drawbacks       | Expected cost |
|-------------------|--------|--------------|--------------------|--------------|----------------------------------|---|---------------|
| Blood             | ++++   | ++           | +                  | ++           | +++                              | slaughterhouse by-product               | ++            |
| Milk              | ++++   | +++          | ++                 | ++           | ++                               | females only                            | +++           |
| Egg white         | ++++   | +++          | ++                 | +            | ++                               | females only, transgenics not available | +++           |
| Silk gland        | +      | +++          | ++                 | +            | +                                | easy collection                         | +             |
| Insect haemolymph | +      | ++           | ++                 | ++           | +                                | easy collection                         | ++            |
| Organ extracts    | +      | +            | +                  | ++           | ++++                             | slaughterhouse by-product               | +             |

health of these animals. This is indeed the case for growth hormone which induces gigantism, diabetes and many other health troubles in transgenic mice, pigs and sheeps (Pursel et al., 1989). Moreover, blood is a relatively complex biological fluid. Preparing a recombinant protein from plasma may be not easy. It may also be particularly difficult to separate the human recombinant protein from the corresponding endogenous molecule. Blood can, however, be an interesting source of recombinant proteins in some particular cases. Massoud et al. (1991) were able to obtain transgenic rabbits expressing the human  $\alpha_1$ -antitrypsin gene in their liver. The human protein was found in the blood of the animals at a concentration as high as  $1 \text{ mg ml}^{-1}$ . Such an accumulation was possible not only because the transgene worked actively, but certainly also because this protein is naturally highly stable in blood and present at an elevated concentration in blood of animals. It is also for this reason that the transgenic rabbits were perfectly healthy. The human  $\alpha_1$ -antitrypsin extracted from the blood of the rabbits was glycosylated and biologically active. Another striking example is the case of human haemoglobin which can be synthesized in an active form in the red blood cells of transgenic pigs (Swanson et al., 1992). Human factor VIII was also found, although at a relatively low level, in the blood of transgenic mice (Mikkelsen et al., 1992). Interestingly, a DNA fragment containing the core region of human immunoglobulin  $\kappa$  locus gave rise to the secretion of functional antibodies in transgenic mice obtained from ES cells previously transfected with a YAC vector containing the foreign DNA (Davies et al., 1993).

Egg white is thought to be an excellent source of recombinant proteins. It is abundant, easily collected and the strong promoter of ovalbumin gene should be able to very efficiently drive the expression of a foreign gene in the oviduct of transgenic chicken. Although attractive, this system has gained little attention so far. This is obviously due to the fact that it remains a very difficult task to prepare transgenic birds for technical reasons (Shuman, 1991). Yolk, although also abundant, cannot so easily be the source of recombinant proteins. Indeed, vitellogenin is syn-

thesized in liver, transported by blood, absorbed by the oviduct where it contributes to form yolk.

The secretions of silk gland and insect haemolymph are possible candidates as sources of recombinant proteins (Maeda et al., 1985; Rancourt et al., 1990). The availability of these biological fluids is, however, limited, and it is by no means certain that the recombinant proteins elaborated by the cells of these non-vertebrates will be properly matured.

It may happen that a given protein is properly matured in only a very limited number of cell types of an animal. In such a situation, the recombinant molecule has little chance of being present in blood or in another abundant and easily collectable biological fluid in sufficient amounts to be purified at an industrial scale. In such cases, it is conceivable to collect organs generating the recombinant proteins in their appropriate form from the transgenic animals in slaughterhouses and to start to purify the proteins from these tissues.

At present, milk appears to be the best source of recombinant proteins. It is very abundant, very rich in proteins, easily collected, and not expected to contain toxic substances. Mammary gland secretion is directed essentially towards the outside of the body, being devoted to offsprings. A recombinant protein secreted in milk is, therefore, not expected to interfere greatly with the metabolism of the lactating female. A few years ago, Simons et al. (1987) gave a direct demonstration that this method is most likely amenable to the industrial area when they observed that transgenic mice secreted up to  $23 \text{ mg ml}^{-1}$  of ovine  $\beta$ -lactoglobulin in their milk. For these reasons, most of the efforts to use transgenic animals as the source of recombinant proteins lie presently with milk.

#### 4. Which vectors to use

##### 4.1. The available protein gene regulatory regions

As mentioned above, the vectors to be used for directing the expression of transgenes in a given tissue or in all tissues must contain the appropri-

ate regulatory regions. Many of such DNA sequences are now available, showing acceptably good specificity for a given cell type and, in some cases, a high potency. In cases of recombinant proteins being prepared in the milk of transgenic animals, the regulatory regions from milk protein genes must be used (Fig. 1). These genes are expressed at high levels exclusively in the mammary gland of lactating animals. Their regulatory regions are thus expected to direct the expression of a foreign gene specifically in this tissue. The major specific milk proteins are  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ - and  $\kappa$ -caseins present in most species at various concentrations,  $\beta$ -lactoglobulin present essentially in ruminant milk,  $\alpha$ -lactalbumin synthesized in most species, and whey acidic protein (WAP) found essentially in rodent milk. These genes are studied for several reasons: to know their structure to improve animal genetic selection, to use them to tentatively change milk composition (to improve cheese yield or to humanize milk), to use their promoters to produce recombinant protein in milk and, of course, to do fundamental studies on the hormonal control of their expression. The milk protein genes from different species inducing human, cow, goat, sheep, pig, camel, rabbit, guinea pig, rat, mouse and even marsupials are being studied (Campbell et al., 1984; Qasba and Safaya,

1984; Jones et al., 1985; Hall et al., 1987; Gorodetski et al., 1988; Ali and Clark, 1988; Vilotte et al., 1989; Thépot et al., 1990, 1991; Devinoy et al., 1991; Koczan et al., 1991; Jolivet et al., 1992a,b; Persuy et al., 1992; Lee and Oka, 1992; Groenen et al., 1992; Schmidhauser et al., 1992; Roberts et al., 1992). A relatively large number of DNA sequences to be used for the generation of vectors expressing foreign genes, specifically in the mammary gland, are thus available.

#### 4.2. The level of transgene expression in milk

Experiments carried out by the various groups are listed in Tables 4–6. From these data it appears that when fragments of genomic DNA containing the whole milk protein genes were used to generate transgenic animals, the expression of the transgenes was very high in several cases, reaching the level observed with endogenous genes (Table 4). With gene constructs containing the whole foreign gene and a milk protein gene regulation sequence, the level of expression was high in some cases and very low in others. It was particularly high in the case of human  $\alpha_1$ -antitrypsin gene in mouse and sheep using ovine  $\beta$ -lactoglobulin gene promoter (Archibald et al.,

Table 4  
Expression of whole milk protein genes from various origins in transgenic animals

| Gene                         | Transgenic animal | Protein (mg ml <sup>-1</sup> ) | Ref.                  |
|------------------------------|-------------------|--------------------------------|-----------------------|
| Ovine- $\beta$ lactoglobulin | mouse             | 23                             | Simons et al., 1987   |
| Ovine- $\beta$ lactoglobulin | mouse             | 0–23                           | Harris et al., 1990   |
| Rat- $\beta$ casein          | mouse             | a few                          | Lee et al., 1988      |
| Goat- $\beta$ casein         | mouse             | 24                             | Persuy et al., 1992   |
| Goat- $\beta$ casein         | mouse             | 1                              | Roberts et al., 1992  |
| Bovine- $\alpha$ lactalbumin | mouse             | 0.45                           | Vilotte et al., 1992  |
| Bovine- $\alpha$ lactalbumin | mouse             | 3.7                            | Soulier et al., 1992  |
| Rat WAP                      | mouse             | a few                          | Bayna and Rosen, 1990 |
| Rat WAP                      | mouse             | a few                          | Dale et al., 1992     |
| Mouse WAP                    | pig               | 1                              | Wall et al., 1991     |
| Mouse WAP                    | pig               | 1                              | Shamay et al., 1991   |
| Mouse WAP                    | pig               | 1                              | Shamay et al., 1992a  |
| Mouse WAP                    | pig               | 1                              | Shamay et al., 1992b  |
| Mouse WAP                    | mouse             | not measured                   | Burdon et al., 1991a  |
| Mouse WAP                    | mouse             | not measured                   | Burdon et al., 1991b  |

The values indicated as mg ml<sup>-1</sup> of milk represent the highest concentration found in all the animals of a given experiment.

1990; Wright et al., 1991) of human and bovine growth hormone and the Arg 358 mutant  $\alpha_1$ -antitrypsin genes (Bischoff et al., 1992; Devinoy et al., 1993). It was very low with the human PS2 gene using the mouse WAP promoter (Tomasetto et al., 1989) and with the human erythropoietin using the rabbit WAP promoter (Attal et al., unpublished data). When foreign cDNA were used rather than whole genes, the expression was low in almost all cases. Curiously, the human protein C was expressed at a relatively high level ( $\approx 1$  mg ml<sup>-1</sup>) in the milk of transgenic pigs and very poorly in transgenic mice, although the mouse WAP promoter was in the gene construct (Velandier et al., 1992).

From the data, it is difficult to draw clear general rules to construct efficient vectors for expression of foreign genes in milk. Most likely, the regulation regions from the various genes are not of equal potency. This is probably the case for the WAP promoters. Indeed, the mouse WAP gene promoter was rather weak when associated with the whole human growth hormone gene (Reddy et al., 1991; Tojo et al., 1993), whereas the rabbit WAP gene promoter was particularly potent with the same gene (Devinoy et al., 1993). The rabbit promoter region used contained a 6.3-kb DNA fragment, whereas the mouse promoter region only used a 2.6-kb DNA fragment. An essential stimulatory element was found at about 6 kb in the rabbit promoter (Devinoy et al., 1991). This element is most likely not present in

the mouse promoter, which might explain its relative weakness. As a general rule, the regulatory elements involved in the control of milk protein gene expression are still far from being known in detail. The gene constructs using these promoters are therefore still done empirically, leading to unpredictable success and failure.

#### 4.3. The role of introns in transgene expression

The regulatory elements located upstream of the transcribed regions of the genes are of course not the only controlling factors in the overall expression of a gene. The different elements which may interfere with the gene transcription, the mRNA stabilization and translation are depicted in Fig. 2. Introns which are often of no use in expressing foreign genes in cultured cells, even when integrated, are essential in most cases in transgenic animals (Brinster et al., 1988). Obviously, introns do not all have the same intrinsic potency. The intron SIS formed with one part of an immunoglobulin gene intron and another part of an adenovirus gene intron showed very high potency (Choi et al., 1991), whereas the SV40 small t intron is not fully functional due to its extremely small size (Fu and Manley, 1987; Huang and Gorman, 1990). However, the intrinsic potency of an intron is not the sole factor responsible for its activity when added in a gene construct. Indeed, a systematic study carried out by Palmiter et al. (1991) revealed that a given intron

Table 5  
Expression of foreign whole genes in the milk transgenic animals

| Regulation region            | Foreign gene                          | Transgenic animal | Secreted protein (ml <sup>-1</sup> ) | Ref.                     |
|------------------------------|---------------------------------------|-------------------|--------------------------------------|--------------------------|
| Ovine- $\beta$ lactoglobulin | human- $\alpha_1$ antitrypsin         | mouse             | 7 mg                                 | Archibald et al., 1990   |
| Ovine- $\beta$ lactoglobulin | human- $\alpha_1$ antitrypsin         | sheep             | 35 mg                                | Wright et al., 1991      |
| Mouse WAP                    | human PS2                             | mouse             | 1.5 $\mu$ g                          | Tomasetto et al., 1989   |
| Mouse WAP                    | human GH                              | mouse             | 500 $\mu$ g                          | Reddy et al., 1991       |
| Mouse WAP                    | human GH                              | mouse             | a few $\mu$ g                        | Günzburg et al., 1991    |
| Rabbit WAP                   | human GH                              | mouse             | 22 mg                                | Devinoy et al., 1993     |
|                              |                                       |                   |                                      | Stinnakre et al., 1992   |
| Rabbit WAP                   | bovine GH                             | mouse             | 8 mg                                 | Devinoy et al., in press |
| Rabbit WAP                   | human- $\alpha_1$ antitrypsin-Arg 358 | mouse             | 10 mg                                | Bischoff et al., 1992    |

The concentration of the foreign proteins in milk shown in the table are the highest found in the animals of a given experiment.

explain its relationship, the regulatory of milk protein being known in these promoters usually, leading to

#### Gene expression

located upstream of genes are of course; in the overall different elements of transcription, translation are determined often of no use in cultured cells, even in most cases in (al., 1988). Obviously the same intrinsic with one part of and another part showed very high whereas the SV40 functional due to its (ley, 1987; Huang the intrinsic position factor responsible in a gene construct carried out by at a given intron

may or may not be efficient according to the cDNA and the promoter to which it is associated. It seems, therefore, that introns contain multiple signals of unknown nature which govern the status of the gene during development. The association of a given intron with the rest of the gene construct must also create cryptic signals, case by case, which modify more or less heavily the expression of the resulting transgene. Therefore, in most cases the presence of at least one intron seems to be compulsory to obtain a good expression of a transgene. However, in this respect no general rules can be edicted as long as more combinations will have been obtained and examined. Unfortunately, cultured cells provide only limited information about the efficiency of a gene construct to be used in transgenic (Brinster et al., 1988).

The mechanisms leading to elimination of intron sequences from pre-mRNAs are not fully understood, but important facts have been reported. Proteo-ribonuclear complexes are in-

volved in splicing. These complexes recognize sequences along the mRNA which are located in both sides of the internal exons. An internal exon must, therefore, not exceed 300 nucleotides in length to allow association of the splicing complex with the pre-mRNA (Robberson et al., 1990).

The first and last introns seem to be properly withdrawn only if the proteo-ribonuclear complexes recognize the CAP structure and the AAUAAA polyadenylation signal, respectively (Ohno et al., 1987; Niwa and Berget, 1991). A specific consensus sequence may also greatly contribute to eliminating a given intron when present in the following exon (Watakabe et al., 1993). The insertion of a foreign cDNA into a gene which proved efficient in transgenesis turned out to give very disappointing results in most cases (McClenaghan et al., 1991). This strategy relies on the idea that a DNA sequence which showed high efficiency as a transgene must contain indispensable elements for expression. The insertion of a foreign cDNA is not expected to alter this

Table 6  
Expression of foreign cDNAs in the milk of transgenic animals

| Regulatory region            | Foreign cDNA                  | Transgenic animals | Protein (ml <sup>-1</sup> ) | Ref.                       |
|------------------------------|-------------------------------|--------------------|-----------------------------|----------------------------|
| Goat- $\beta$ casein         | human CFTR                    | mouse              | a few $\mu$ g               | Di Tullio et al., 1992     |
| Bovine- $\alpha$ casein      | ovine trophoblastin           | mouse              | 1 $\mu$ g                   | Stinnakre et al., 1991     |
| Bovine- $\alpha_1$ casein    | human lactoferrins            | cow                | ?                           | Krimpenfort et al., 1991   |
| Ovine- $\beta$ lactoglobulin | human serum albumin           | mouse              | 2.5 mg                      | Shani et al., 1992         |
| MMTV                         | bovine- $\alpha_1$ casein     | mouse              | 0.3 mg                      | Yom et al., 1993           |
| Ovine- $\beta$ lactoglobulin | human factor VIII             | sheep              | ?                           | Halter et al., 1993        |
| Ovine- $\beta$ lactoglobulin | human $\gamma$ -interferon    | mouse              | 20 ng                       | Dobrovolsky et al., 1993   |
| Bovine- $\alpha_1$ casein    | human t-PA                    | mouse and rabbit   | 50 $\mu$ g                  | Riego et al., 1993         |
| Mouse WAP                    | human $\beta$ -interferon     | mouse              | ?                           | Schellander and Péli, 1992 |
| Mouse WAP                    | human t-PA                    | mouse              | 460 ng                      | Gordon et al., 1987        |
| Mouse WAP                    | human t-PA                    | mouse              | 460 ng                      | Pittius et al., 1988       |
| Mouse WAP                    | human t-PA                    | goat               | 3 $\mu$ g                   | Ebert et al., 1991         |
| Mouse WAP                    | human CD4                     | mouse              | 200 ng                      | Yu et al., 1989            |
| Mouse WAP                    | human protein C               | pig                | 1 mg                        | Velander et al., 1992      |
| Ovine- $\beta$ lactoglobulin | human factor IX               | sheep              | 25 ng                       | Simons et al., 1988        |
| Ovine- $\beta$ lactoglobulin | human factor IX               | sheep              | 25 ng                       | Clark et al., 1989         |
| Ovine- $\beta$ lactoglobulin | human $\alpha_1$ -antitrypsin | mouse and sheep    | 18 mg                       | McClenaghan et al., 1991   |
| Bovine- $\alpha_1$ casein    | human urokinase               | mouse              | 2 mg                        | Meade et al., 1990         |
| Rabbit- $\beta$ casein       | interleukin-2                 | rabbit             | 430 ng                      | Bülher et al., 1990        |
| Rat- $\beta$ casein          | human FSH                     | mouse              | 15 $\mu$ g                  | Greenberg et al., 1991     |

The concentration of the foreign proteins in milk are the highest found in the animals of a given experiment.

ald et al., 1990  
t et al., 1991  
setto et al., 1989  
y et al., 1991  
urg et al., 1991  
oy et al., 1993  
ikre et al., 1992  
oy et al., in press  
off et al., 1992  
f a given experiment.

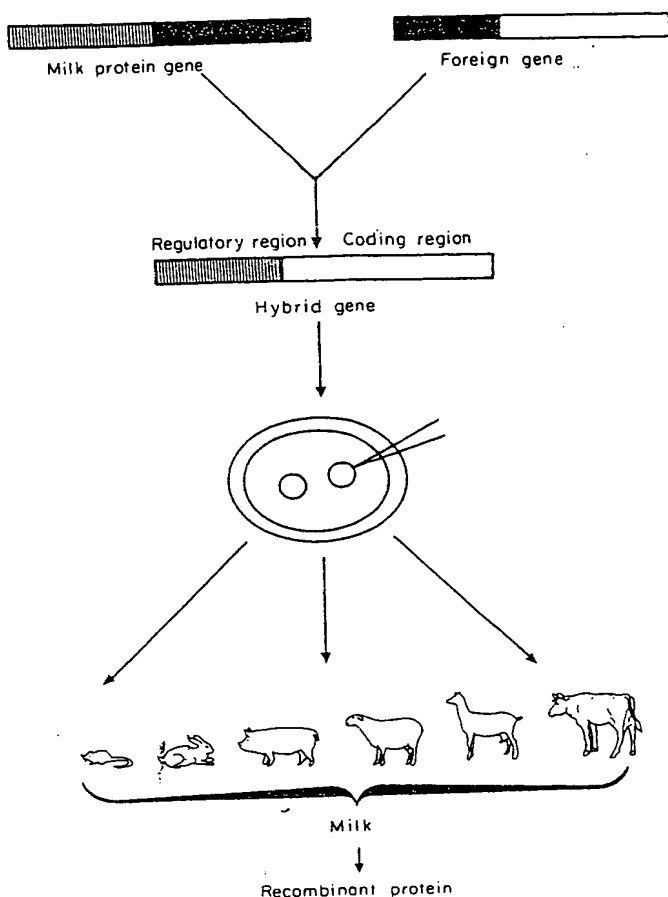


Fig. 1. Schematic representation of the generation of vectors for the expression of foreign genes in the milk of transgenic animals. The regulatory region from a milk protein gene can be fused to the transcribed region of a gene of interest. The resulting gene construct can be injected into embryo pronuclei to generate transgenic mammals of various sizes which are expected to express the foreign gene in their milk.

efficiency. In practice, the reverse is in most cases the reality. Generally speaking, ideally a whole gene with its own elements selected during evolution should therefore be used rather than the corresponding cDNA. Unfortunately, in some cases only cDNA are available or in practise amenable to easy manipulation. This is true for

factor VIII gene which is as long as 250 kb. The low level of expression obtained when cDNAs are introduced into whole genes used as vectors is most likely due to the fact that the above-reported rules have not been respected.

#### 4.4. The influence of untranslated regions of mRNA

Essentially the same reasoning is true for the other elements of the gene constructs. The 5' untranslated region (5' UTR, also named leader) may favour more or less translation. A minimum length of 77 nucleotides is required to obtain maximum efficiency (Kozak, 1991). A leader sequence rich in GC is generally less efficient than another richer in AU (Kozak, 1991), the GC structures being more stable, preventing the ribosomes to reach the initiator AUG with good efficiency (Gallie et al., 1987). The 5' UTR may even contain major elements drastically controlling the translation of the mRNAs. This is the case for several iron-binding proteins which have a regulatory element activated by the metal ion (Klausner and Harford, 1989).

The 3' UTR of mRNA participate in some cases in the stabilization of the mRNA. The mRNA for some house-keeping genes and particularly for some oncogenes have AU-rich sequences in their 3' UTR which make them fragile towards nucleases. Quite different mechanisms are in fact involved in the control of mRNA stability (Cleveland and Yen, 1989). These facts have to be taken into consideration in the construction of a vector for transgenesis.

#### 4.5. The influence of transcription terminator

Transcription termination is carried out through mechanisms which have not been completely elucidated (Edwards-Gilbert et al., 1993). Empirically, it appears that different terminators have quite different intrinsic potency. The termi-

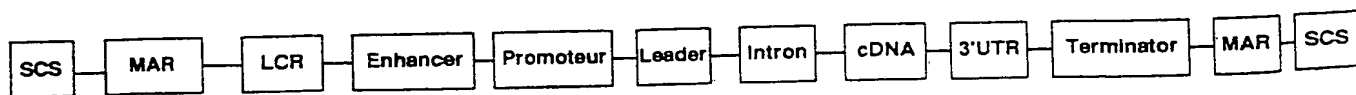


Fig. 2. Schematic representation of the various elements which should be present in a vector to optimize the expression of a transgene. For details see the text.



nator of SV40 late gene is thus 4-times more potent than its early gene counterpart (Carswell and Alwine, 1989). A short DNA sequence ( $\approx 70$  bp) in the mouse WAP gene terminator appeared more efficient than a longer one of several kb in transgenic mice, whereas the late gene SV40 terminator, which is highly potent in cultured cells, worked very poorly in these conditions (Dale et al., 1992). The human erythropoietin gene contains in its terminator region a silencer which is more or less activated according to oxygen tension (Semenza and Wang, 1992). Choosing an efficient transcription terminator cannot at present be done following logical rules. Moreover, it would not be surprising if a given terminator exhibits quite different efficiency according to the construct in which it is inserted.

#### 4.6. The influence of transgene insertion site

Experiments carried out with transgenic animals soon revealed that in the great majority of cases, a transgenic expression is heavily dependent on its site of integration in the host genome rather than on the number of copies. The site of integration of a transgene is presently unpredictable when the DNA is microinjected into the pronucleus by the conventional method. This largely explains why the expression of a transgene in a given animal is also unpredictable. This observation was done repeatedly with transgenes expressed in the milk with one clear and noticeable exception. The whole  $\beta$ -lactoglobulin gene was not only expressed at a high rate in transgenic mice, but also as a function of its copy number (Whitelaw et al., 1992). This DNA fragment might thus contain a MAR or a SCS (see below). The fact that the expression of a given transgene is governed by its chromatin environment has been ascertained by experiments in which a given transgene, weakly and strongly expressed in different mice, was isolated from these animals and reinjected into new mouse embryos. The resulting transgenic animals expressed their transgene at a high and low level when they received the efficient and inefficient mouse genomic DNA containing the transgene, respectively (Al Shawi et al., 1990).

The idea which is generally accepted is, therefore, that the transgene must be located in a highly transcribed open region of the chromatin to work properly. Apart from being obtained by chance, such a situation may be encountered, at least to some extent, using appropriate vectors. Genomic DNA fragments which were able to allow a good expression of a given transgene (Al Shawi et al., 1990) might be used to construct vectors. This rather heavy approach does not seem to have been retained so far. Some genes isolated or belonging to a given group of genes are controlled by locus control regions (LCR). This is the case for the human  $\beta$ -globin locus. Such a DNA sequence is located far upstream of the globin genes. This sequence, which is DNase I hypersensitive, must be added to the isolated globin genes from this locus to allow their expression as transgenes (Stamatoyannopoulos, 1991).

The  $\beta$ -globin LCR acts as a super enhancer which strongly stimulates the transgenes which are then expressed according to their copy number. Until now only a very limited number of LCR have been identified. Each LCR seems to be specific in a given locus and it is by no means certain that each gene or group of genes is dependent on such regulatory elements. The whole bovine casein locus can be isolated in DNA fragments not longer than 200 kb (Ferretti et al., 1990; Threadgill and Womack, 1990). The presence of an LCR specific in this casein locus has not been reported so far. Such a sequence inserted into vectors might, of course, improve specifically the expression of foreign genes in milk.

Chromatin is bound to the nuclear structure called matrix, forming loops. DNA is linked to the nuclear matrix through sequences named matrix attached regions (MAR) or scaffold attached regions (SAR). Many, but not all, of these sequences are AT rich (Dworetzky et al., 1992; Jankelevich et al., 1992). Some of them are involved in the control of DNA replication and segregation (DePamphilis, 1993). Others (or possibly the same MAR) play an essential role in controlling gene expression (Bonifer et al., 1991; Zhao et al., 1993). Some MARs have been proven to have the capacity to insulate a gene and a

transgene from their chromatin environment leading to a high expression which becomes dependent of their copy number (Bonifer et al., 1990). MAR may thus be added on one end, and perhaps preferably on both ends, of a gene construct (one MAR may perhaps be sufficient when concatemers of the injected material are formed before its integration. In these conditions, the transgene becomes surrounded by MAR). A coinjection of conventional gene constructs and MAR may lead to the generation of transgenics harbouring the foreign gene in the vicinity of the cointegrated MAR. This cointegration of the foreign DNA and the MAR results in a significant improvement in the expression of the transgene (McKnight et al., 1992). This simple procedure does not oblige the experimentators to introduce in vitro MAR sequences in their gene constructs. The exact role of MAR is in fact presently somewhat confusing. The presence of an AT-rich region with the ATTA and ATTTA consensus sequences appear to favour greatly the expression of downstream genes (Bode et al., 1992; Boulikas, 1993; Schöffl et al., 1993). However, an AT-rich consensus sequence which is capable of binding strongly to nuclear matrix may be unable of stimulating the expression of a transgene. Such a situation was observed when the MAR sequence located in the downstream region of the human apolipo-protein B100 (Levy-Wilson and Fortier, 1989) was added in front of the rabbit WAP or the cytomegalovirus early genes promoters (Attal et al., unpublished observations).

Recent data suggest that sequences capable of insulating genes and transgenes are located in the upstream region of the human and chicken  $\beta$ -globin locus (Chung et al., 1993) and of the mouse metallothionein gene (Palmiter et al., 1993). These sequences seem to not necessarily have nuclear matrix-binding capacity, and they show strong functional analogy with AT-rich regions named SCS (specialized chromatin structure) which have been found in drosophila genome and which strongly insulate transgenes (Kellum and Schedl, 1991; Farkas and Udvardy, 1992).

SCS regions rather than MAR sequences should therefore be added at the beginning and

perhaps at the end of a gene construct to generate efficient insularity of transgene. Confusion may have come from the fact that the A element located in the upstream region of chicken lysozyme gene has the capacity to insulate a transgene (Bonifer et al., 1990), while also being a MAR (von Kries et al., 1990).

An open configuration of chromatin in the vicinity of a given transgene can be obtained by coinjection of the gene construct of unknown efficiency and a gene known to become regularly an efficient transgene. Using this simple method, a construct containing the  $\beta$ -lactoglobulin promoter fused to the human  $\alpha_1$ -antitrypsin cDNA became better expressed in transgenic mice when injected with the entire ovine  $\beta$ -lactoglobulin gene (Clark et al., 1992). It is not known whether a sequence expressed specifically at a high level in mammary cells is required to obtain this rescuing of transgene expression or whether any highly expressed sequence might have the same effect. One may hypothesize that a gene not specifically expressed in milk will stimulate the expression of the gene construct but also will be responsible for a significant expression in tissues other than the mammary gland.

Vectors, the YAC, being expressed in yeast, can harbour very long fragments of DNA, up to several hundreds of base pairs. The YAC can thus be used to generate vectors containing long fragments of genomic DNA surrounding milk protein genes. These long fragments have a good chance of containing most, if not all, the elements which control the expression of the genes and the transgenes. YAC constructs proved to be utilisable in generating transgenic mice which express their transgenes in a satisfactory manner (Capecchi, 1993; Davies et al., 1993).

#### 4.7. Use of embryonic stem cells (ES) to generate transgenic animals

Embryonic stem cells can be cultured and reintroduced into developing embryo and participate to the generation of chimaeric animals. Foreign genes can be introduced with a homologous recombination into the genome of the ES cells before they are mixed with the cells of the devel-

O.  
P  
O.  
D  
P  
di  
H  
m  
P  
br  
ge  
re  
wh  
er

5.

sex  
rec  
na  
dal  
yea  
cor  
the  
run  
day  
sign  
with  
199  
at a  
pro  
the  
cee

Tabl  
Com  
Spec

Mous  
Rabb  
Pig  
Sheep  
Cow

oping embryo (Bradley et al., 1992). This complex procedure leads to the precise replacement of one gene by another. In this manner, a foreign DNA sequence can be introduced within a milk protein gene. As expected, the foreign gene introduced with this method will be well-expressed. However, this approach remains restricted to mice, ES cells from other species being not presently available. The possibility of using embryonic germ cells (EG cells) rather ES cells to generate chimaeric animals (McLaren, 1992) has recently opened new avenues for farm animals which are the only species to be potential producers of recombinant proteins at an industrial scale.

### 5. Which mammals to use

Mammals vary quite differently in size, and several of them have been chosen to produce recombinant proteins in their milk. Ruminants, namely goat and sheep, appear the best candidates to produce proteins up to several tons per year (Ebert et al., 1991; Wright et al., 1991). Pig is considered as a possible living fermentor, although milk cannot be collected as easily as from ruminants (Velandar et al., 1992).

Rabbit produces up to 200–250 ml of milk per day. Its milk is particularly rich in protein and a significant proportion of milk can be obtained with a simple device (Lebas, 1970; Duby et al., 1993). Transgenic rabbits can be easily obtained at a relatively low cost. This species is also highly prolific and it is therefore a good candidate for the production of recombinant proteins not exceeding 1 kg per year.

Table 7

The potential production of recombinant proteins in the milk of different transgenic mammals

| Animal                 | Amounts of proteins to produce in milk |
|------------------------|--|
| Mouse                  | up to 1 g                              |
| Rabbit                 | 1 kg a <sup>-1</sup>                   |
| Sheep,<br>goat,<br>pig | 1–1000 kg a <sup>-1</sup>              |
| Cow                    | several t a <sup>-1</sup>              |

Mice do not easily deliver their milk. Incubating the isolated mammary gland on ice for a few hours leads to a complete secretion of the milk it contains. Undegraded and biologically active human growth hormone was obtained in this way (about 1.5 ml per mouse; Stinnakre et al., 1992). Following this procedure, 100 lactating mice might provide a few hundred mg of a recombinant protein, a quantity which is sufficient to study the biochemical and some of clinical properties of the protein. Transgenic mice can therefore be considered not only as laboratory animals to evaluate the efficiency of gene constructs to be used in larger species but as a true 'living mini-fermentor'.

Cow is probably the only mammalian species potentially capable of synthesizing the 400 t of human albumin which are needed each year (Table 7). Generating transgenic cows using the conventional gene microinjection is a very burdensome and costly task. This is due to the fact that much less embryo can be obtained in a given period of time by the conventional superovulation and in vivo fertilization classically used in mouse.

Table 8

Comparison of transgenic yield in different species

| Species | Number of embryos per superovulation | Rate of pregnancy (%) | Number of donors per recipient | Rate of born embryos (%) | Transgenic animals (% of born) | Number of females per transgenic | Months to obtain F2 |
|---------|--------------------------------------|-----------------------|--------------------------------|--------------------------|--------------------------------|----------------------------------|---------------------|
| Mouse   | 15                                   | 50                    | 2                              | 10–20                    | 15                             | 10                               | 7.5                 |
| Rabbit  | 20                                   | 50                    | 2                              | 10                       | 10                             | 15                               | 17                  |
| Pig     | 15                                   | 40                    | 2                              | 5–8                      | 10                             | 20                               | 38                  |
| Sheep   | 4                                    | 40                    | 1–5                            | 15                       | 5                              | 40                               | 52                  |
| Cow     | 5                                    | 20                    | 1                              | 10                       | 5                              | 40                               | 100                 |

The yield of transgenic calves is also generally very low in comparison to other species (Table 8).

It has become possible to obtain one cell embryos through an all in vitro protocol. This method includes the following steps: collection of ovaries from females at any physiological stage in slaughterhouse, in vitro maturation of oocytes isolated from the ovaries, and in vitro fertilization of the oocytes. The availability of embryos is considerably increased with this procedure. Consequently their cost is dramatically reduced. This method has been defined and even used in cow (Krimpenfort et al., 1991). It has been extended to sheep and is being adapted to goat. After the gene microinjection, the cow embryos can be cultured up to the blastocyst stage. Only the embryos surviving the manipulation reach this stage. A detection of the transgene in a few cells explanted from the blastocytes can be performed using the PCR technique, although with somewhat limited reliability (King and Wall, 1988; Ninomiya et al., 1989; Burdon and Wall, 1992). Using all these techniques, no more donor females are required to obtain embryos and a minimum number of recipients are needed to develop the embryos considered as transgenic after the PCR test. Thanks to these techniques, transgenesis in farm animals has been greatly facilitated and the use of milk as the source of recombinant has consequently become closer to reality.

## 6. What is the future of the 'living fermentors'?

Predicting what will be the real future of living fermentors is not easy. A few years ago several reports indicated that by the end of this century 10% of the recombinant proteins would be extracted from the milk of transgenic animals, representing an annual market of approx. 100 million dollars (Genetics Technology News, 1990; Technology Management Group, 1990). The reality is that there is still no protein prepared from transgenic animals on the market. Human  $\alpha_1$ -antitrypsin is well on the way and protein C might follow. It seems, therefore, hard to believe that the situation described above will be encountered so quickly. Indeed several problems remain not

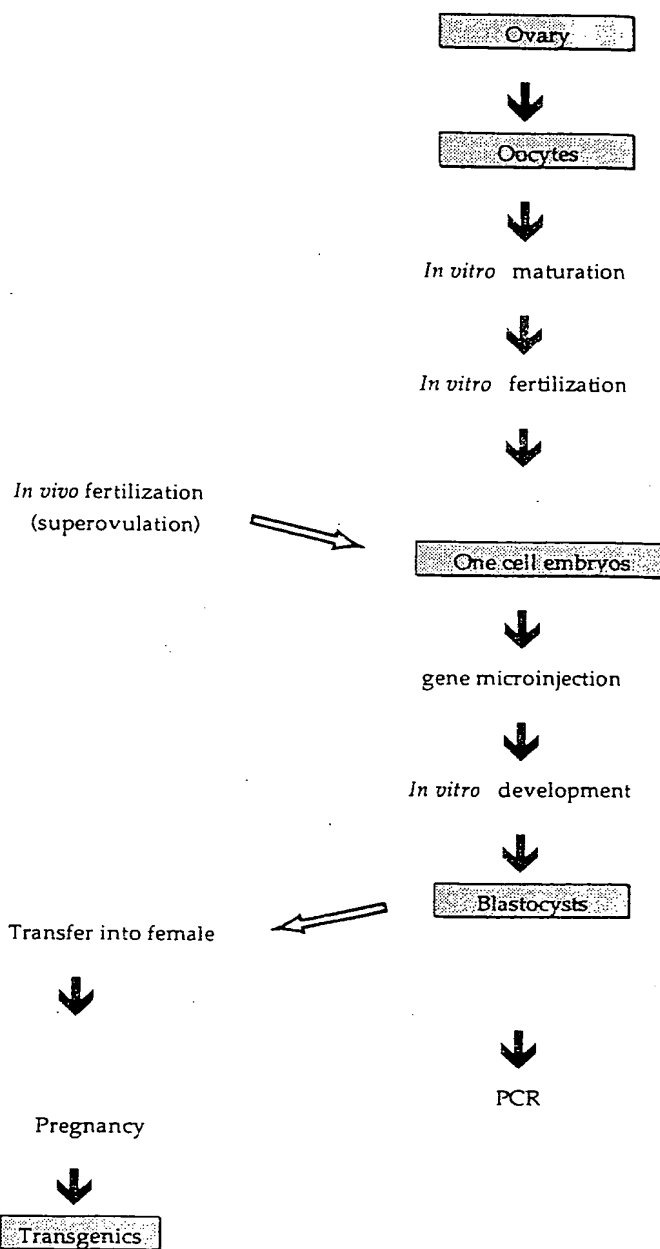


Fig. 3. Schematic representation of the different steps of the protocol leading to the preparation of mature oocytes, one cell embryos and blastocysts in vitro. For details see the text.

completely solved. Transgenesis in farm animals is being greatly improved by the new techniques of embryo manipulation (Fig. 3). Obtaining transgenic farm animals will, however, remain a rather hard task with relatively long delays between the time when a gene construct is available and the

moment a significant number of animals can be milked. The possibility to clone the embryo through nuclear transfer (Yang and Anderson, 1992) will help to shorten the delay, allowing a by-passing of a part of the reproduction cycle.

The construction of efficient vectors turned out to be more complicated than anticipated. This is due to the fact that the problem is in itself complex. During embryo development a transgene is subjected to many events which may lead to an inactivation of the chromatin region in which it is integrated. Testing new constructs to be expressed in milk is a slow process. Indeed, cell cultures are of little help for this purpose (Brinster et al., 1988) and transgenic animals must be obtained to test each vector. Only transgenic females can be directly used and the information concerning the vectors becomes available only when they are mature and lactate. In fact, a relatively small number of laboratories seem to be concerned with vectors expressing foreign genes at a high level in transgenic animals. Essentially, people trying to produce recombinant proteins or to express antisense RNA presently need potent vectors. For the great majority of studies using transgenic mice, vectors working modestly are sufficient to express such or such cellular protein of interest. Although the construction of efficient vectors for expression of foreign genes in milk is not a problem solved in all cases, there are some reasons to believe that, in future, perhaps 80% of the transgenes will work in a satisfactory manner. Until this situation becomes a reality, if it does, success is welcome but cannot be predicted.

Purifying foreign proteins from milk does not seem a major problem. Milk is not an extremely complex biological fluid. It does not degrade proteins through proteolysis. A satisfactory biochemical purity of the recombinant proteins seems to be obtained with conventional methods (Ebert et al., 1991; Wilkins and Velander, 1992). In most cases, the recombinant protein is expected to be present in a soluble form in the whey. Interestingly, in one case at least, a hydrophobic protein, the CFTR, was found associated with the membranes surrounding the milk lipid globules (Di Tullio et al., 1992). The hydrophobic proteins can

thus be recovered from milk fat. Most likely, however, their content will be much lower than that of water-soluble proteins, the surface of the lipid globule membranes harbouring the recombinant proteins being relatively limited.

The biological purity of the recombinant proteins extracted from milk will probably be more difficult to ascertain. Virus and prions may still contaminate the purified recombinant proteins. Tests will have to be done to detect such contaminants and the herds of transgenic animals will have to be kept under excellent breeding conditions. It is indeed paradoxical to use genetic engineering to prepare recombinant proteins in order to escape the use of human organ but still to come back to transgenic animals to obtain these molecules. Some long-standing practises incline, however, to be optimistic. Indeed, insulin injected daily to diabetics has long been exclusively extracted from pig pancreas without being highly purified. Still, this hormonal preparation is considered safe, as far as viral contamination is concerned.

It is by no means certain that the mammary epithelial cells are able to proceed to all the desired post-translational modifications. These cells can indeed be O- and N-glycosylated proteins and they are differentiated for efficient secretion of proteins in contrast to most commonly used recombinant cells. However, milk proteins which are glycosylated are known to contain somewhat variable amounts of carbohydrates, as though the glycosylation process could not be quantitative, given the high flow of neo-synthesized proteins which migrate through the Golgi apparatus. Glycosylation of proteins is known to depend on the physiological state of the cells and the microheterogeneity of glycoproteins may modify, to some extent, their biological properties (Goochee and Monica, 1990).

In this respect, however, the recombinant proteins secreted in milk are not expected to be less exploitable than those found in the medium of cells expressing foreign genes. Moreover, it is conceivable to genetically modify the mammary cells through transgenesis with genes coding for glycosylation enzymes to give them the capacity to glycosylate proteins in a more appropriate

manner. Although theoretically feasible, as it is for animal cells in culture and yeast, such cellular modifications may, in practise, more or less profoundly disturb the cell physiology.

The post-translational modifications of proteins are not the only problem which may limitate the use of transgenic animals to produce recombinant proteins. The process of migration in the cell and exocytosis of the neosynthesized proteins is complex. It involves the action of Golgi apparatus and it seems that signals within the protein structures more or less favour the migration of proteins towards the outside of the cells (Edington, 1992). In this respect, it is interesting to note that the human factor VIII in its  $\Delta$ II deleted form is more readily secreted than the natural molecule, as though some sequences of these proteins contributed to slow its exportation out of the cells (Meulien et al., 1988).

Although milk is secreted in a specific compartment not directly in contact with blood circulation, one may expect that some of the recombinant proteins will cross the mammary epithelium. Indeed, caseins are not present in blood during lactation but a protein like WAP is obviously crossing the mammary epithelium and it is found at a significant level in the circulation (Grabowski et al., 1991). It is conceivable that only the smallest proteins will pass through the epithelium, but in these cases, the transgenic animals may be profoundly disturbed by the presence of an active recombinant protein in their blood when they start lactating. One may thus predict that, in a certain number of cases, the animals will not be utilisable. Lactation itself may be profoundly altered by the product of the transgene as it is for WAP expressed too early, during pregnancy in mouse and pig (Burdon et al., 1991a,b; Shamay et al., 1992a,b).

On the other hand, most of the transgenes show a background of aspecific expression in tissues other than those in which they should be expressed. This is also the case for foreign genes fused to milk protein gene promoters. It is generally admitted that this is due to the fact that the transgenes are integrated in the host genome in the vicinity of endogenous regulatory regions which reduce more or less their specificity of

expression. This problem may be solved by choosing the transgenic lines of animals which show the lowest leaky expression out of the mammary gland. The addition of SCS in the vectors may also contribute to reduce the aspecific expression of the transgene (McKnight et al., 1992). Whatever happens, the flow-through of some recombinant proteins from milk to blood will, in some cases, remain an inevitable event. A point of some potential importance must also be taken into consideration. Blood or a given organ can be considered as slaughterhouse by-products but only of course if meat remains the major product. Given the difficulties encountered by companies which propose transgenic plants for human consumption, one way predicts that selling the meat from transgenic animals at a normal price will be a real problem, at least the first time. The rejection of genetically modified organisms by some people is undoubtedly in most cases more emotional than reasonable. Reasonably, it is imaginable that acceptance of 'transgenic' meat will increase in the future after becoming more familiar with the situation (Van Brunt, 1990; Fox, 1993a,b). During this transition period, a company may be forced to delay its use of transgenic animals to produce recombinant proteins. The resulting cost may dissuade the use of 'living fermentors' for some time. A similar, although much less acute problem, may be encountered when the recombinant proteins are being produced in milk. Indeed, the consumption of the transgenics by human beings may also be forbidden in this case but the resulting extra costs are expected to be minor, given the relatively reduced number of involved animals.

Improvement of the techniques aiming at producing recombinant proteins from transgenic animals are real and this approach remains very attractive, given its very high potency, once the transgenic animals have been generated. Of course, the real commercial success of this method will be greatly determined by its cost. Indeed, the other biological systems capable of producing recombinant proteins and, in particular, transgenic plants (Hiatt, 1990; Mason et al., 1992; Hamamoto et al., 1993) are also being improved (Hodgson, 1993). An insufficient number of recombinant

proteins of commercial interest has been prepared from animals to allow a precise comparison with the competition systems and particularly with cultured mammalian cells. The course of progress inclines therefore to remain reasonably optimistic.

## References

- Ali, S. and Clark, A.J. (1988) Characterization of the gene encoding ovine beta-lactoglobulin. Similarity to the genes for retinol binding protein and other secretory proteins. *J. Mol. Biol.* 199, 415–426.
- Al-Shawi, R., Kinnaird, J., Burke, J. and Bishop, J.O. (1990) Expression of a foreign gene in a line of transgenic mice is modulated by a chromosomal position effect. *Mol. Cell Biol.* 10, 1192–1198.
- Archibald, A.L., McClenaghan, M., Hornsey, V., Simons, J.P. and Clark, A.J. (1990) High-level expression of biologically active human alpha-1-antitrypsin in the milk of transgenic mice. *Proc. Natl. Acad. Sci. USA* 87, 5178–5182.
- Bayna, E.M. and Rosen, J.M. (1990) Tissue-specific, high level expression of the rat whey acidic protein gene in transgenic mice. *Nucleic Acids Res.* 18, 102977–102985.
- Bischoff, R.J., Degryse, E., Perraud, F., Dalemans, W., Ali-Hadji, D., Thépot, D., Devinoy, E., Houdebine, L.M. and Pavirani, A. (1992) A 17.6 kbp region located upstream of the rabbit WAP gene directs high level expression of a functional human protein variant in transgenic mouse milk. *FEBS Lett.* 305, 265–268.
- Blum, P., Velligan, M., Lin, N. and Matin, A. (1992) DnaK-mediated alterations in human growth hormone protein inclusion bodies. *Bio/Technology* 10, 301–304.
- Bode, J., Kohwi, Y., Dickinson, L., Joh, T., Klehr, D., Mielke, C. and Kohwi-Shigematsu, T. (1992) Biological significance of unwinding capability of nuclear matrix-associating DNAs. *Science* 255, 195–197.
- Bonifer, C., Vidal, M., Grosveld, F. and Sippel, A.E. (1990) Tissue specific and position independent expression of the complete gene domain for chicken lysozyme in transgenic mice. *EMBO J.* 9, 2843–2848.
- Bonifer, C., Hecht, A., Saueressig, H., Winter, D.M. and Sippel, A.E. (1991) Dynamic chromatin: the regulatory domain organization of eukaryotic gene loci. *J. Cell. Biochem.* 47, 99–108.
- Boulikas, T. (1993) Nature of DNA sequences at the attachment regions of genes to the nuclear matrix. *J. Cell. Biochem.* 52, 14–22.
- Bradley, A., Hasty, P., Davis, A. and Ramirez-Solis, R. (1992) Modifying the mouse: design and desire. *Bio/Technology* 10, 534–539.
- Brinster, R.L., Allen, J.M., Behringer, R.R., Gelinas, R.E. and Palmiter, R.D. (1988) Introns increase transcriptional efficiency in transgenic mice. *Proc. Natl. Acad. Sci. USA* 85, 836–840.
- Buckholz, R.G. and Gleeson, M.A.G. (1991) Yeast systems for the commercial production of heterologous proteins. *Bio/Technology* 9, 1067–1072.
- Burdon, T., Sankaran, L., Wall, R.J., Spenser, M. and Hennighausen, L. (1991a) Expression of a whey acidic protein transgene during mammary development. *J. Biol. Chem.* 266, 116909–116914.
- Burdon, T., Wall, R.J., Shamay, A., Smith, G.H. and Hennighausen, L. (1991b) Over-expression of an endogenous milk protein gene in transgenic mice is associated with impaired mammary alveolar development and a milchlos phenotype. *Mech. Dev.* 36, 67–74.
- Burdon, T.G. and Wall, R.J. (1992) Fate of microinjected genes in preimplantation mouse embryos. *Mol. Reprod. Dev.* 33, 436–442.
- Bülher, T.A., Bruyere, T., Went, D.F., Stranzinger, G. and Burki, K. (1990) Rabbit beta-casein promoter directs secretion of human interleukin-2 into the milk of transgenic rabbits. *Bio/Technology* 8, 140–143.
- Campbell, S.M., Rosen, J.M., Hennighausen, L.G., Strech, Jurk, U. and Sippel, A.E. (1984) Comparison of the whey acidic protein genes of the rat and mouse. *Nucleic Acids Res.* 12, 8685–8697.
- Capecchi, M.R. (1993) YACs to the rescue. *Nature* 362, 205–206.
- Carswell, S. and Alwine, J.C. (1989) Efficiency of utilization of the simian virus 40 late polyadenylation site: effects of upstream sequences. *Mol. Cell. Biol.* 9, 4248–4258.
- Choi, T., Huang, M., Gorman, C. and Jaenisch, R. (1991) A generic intron increases gene expression in transgenic mice. *Mol. Cell. Biol.* 11, 3070–3074.
- Chung, J.H., Whiteley, M. and Felsenfeld, G. (1993) A 5' element of the chicken beta-globin domain serves as an insulator in human erythroid cells and protects against position effect in *Drosophila*. *Cell* 74, 505–514.
- Clark, A.J., Bessos, H., Bishop, J.O., Brown, P., Harris, S., Lathe, R., McClenaghan, M., Prowse, C., Simons, J.P., Whitelaw, C.B.A. and Wilmot, I. (1989) Expression of human anti-hemophilic factor IX in the milk of transgenic sheep. *Bio/Technology* 7, 487–492.
- Clark, A.J., Cowper, A., Wallace, R., Wright, G. and Simons, J.P. (1992) Rescuing transgene expression by co-integration. *Bio/Technology* 10, 1450–1454.
- Cleveland, D.W. and Yen, T.J. (1989) Multiple determinants of eukaryotic mRNA stability. *New Biol.* 1, 121–126.
- Dale, T.C., Krnacik, M.J., Schmidhauser, C., Yang, C.L.Q., Bissell, M.J. and Rosen, J.M. (1992) High-level expression of the rat whey acidic protein gene is mediated by elements in the promoter and 3' untranslated region. *Mol. Cell. Biol.* 12, 905–914.
- Datar, R.V., Cartwright, T. and Rosen, C.G. (1993) Process economics of animal cell and bacterial fermentations: a case study analysis of tissue plasminogen activator. *Bio/Technology* 11, 349–357.

- Davies, N.P., Rosewell, I.R., Richardson, J.C., Cook, G.P., Neuberger, M.S., Brownstein, B.H., Norris, M.L. and Brüggemann, M. (1993) Creation of mice expressing human antibody light chains by introduction of a yeast artificial chromosome containing the core region of the human immunoglobulin k locus. *Bio/Technology* 11, 911-914.
- DePamphilis, M.L. (1993) Eukaryotic DNA replication: anatomy of an origin. *Annu. Rev. Biochem.* 62, 29-63.
- Devinoy, E., Malienou-N'Gassa, R., Thépot, D., Puissant, C. and Houdebine, L.M. (1991) Hormonal regulatory elements within the upstream sequences of the rabbit whey acidic protein gene direct CAT reporter gene expression in transfected rabbit mammary cells. *Mol. Cell. Endocrinol.* 81, 185-193.
- Devinoy, E., Thépot, D., Stinnakre, M.G., Fontaine, M.L., Grabowski, H., Puissant, C., Pavirani, A. and Houdebine, L.M. (1994) High levels of production of human growth hormone in the milk of transgenic mice: the upstream region of the rabbit Whey Acidic Protein (WAP) gene targets transgene expression to the mammary gland. *Transgenic Res.*, in press.
- Di Tullio, P., Cheng, S.H., Marshall, J., Gregory, R.J., Ebert, K.M., Meade, H.M. and Smith, A.E. (1992) Production of cystic fibrosis transmembrane conductance regulator in the milk of transgenic mice. *Bio/Technology* 10, 74-77.
- Dobrovolsky, V.N., Lagutin, O.V., Vinogradova, T.V., Frolova, L.S., Kuznetsov, V.P. and Larionov, O.A. (1993) Human gamma-interferon expression in the mammary gland of transgenic mice. *FEBS Lett.* 319, 181-184.
- Duby, R.T., Cunliff, M.B., Belak, J.M., Balise, J.J. and Robl, J.M. (1993) Effect of milking frequency on collection of milk from nursing new zealand white rabbits. *Anim. Biotechnol.* 4, 31-42.
- Dworetzky, S.I., Wright, K.L., Fey, E.G., Penman, S., Lian, J.B., Stein, J.L. and Stein, G.S. (1992) Sequence-specific DNA-binding proteins are components of a nuclear matrix-attachment site. *Proc. Natl. Acad. Sci. USA* 89, 4178-4182.
- Ebert, K.M., Selgrath, J.P., DiTullio, P., Denman, J., Smith, T.E., Memon, M.A., Schindler, J.E., Monastersky, G.M., Vitale, J.A. and Gordon, K. (1991) Transgenic production of a variant of human tissue-type plasminogen activator in goat milk: generation of transgenic goats and analysis of expression. *Bio/Technology* 9, 835-838.
- Edgington, S.M. (1992) Rites of passage: moving biotech proteins through the ER. *Bio/Technology* 10, 1413-1420.
- Edwards-Gilbert, G., Prescott, J. and Falck-Pedersen, E. (1993) 3' RNA processing efficiency plays a primary role in generating termination-competent RNA polymerase II elongation complexes. *Mol. Cell. Biol.* 13, 3472-3480.
- Farkas, G. and Udvardy, A. (1992) Sequence of SCS and SCS' Drosophila DNA fragments with boundary function in the control of gene expression. *Nucleic Acids Res.* 20, 2604.
- Ferretti, L., Leone, P. and Sgarbetta, V. (1990) Long Range Restriction Analysis of the Bovine Casein Genes. *Nucleic Acids Res.* 18, 6829-6833.
- Fox, J.L. (1993a) FDA panel ponders labels for BST-derived foods. *Biotechnology* 11, 656-657.
- Fox, J.L. (1993b) FDA reexamines biotech food policy. *Biotechnology* 11, 656.
- Fu, X.Y. and Manley, J.L. (1987) Factors influencing alternative splice site utilization in vivo. *Mol. Cell. Biol.* 7, 738-748.
- Gallie, D.R., Sleat, D.E., Watts, J.W., Turner, P.C. and Wilson, T.M.A. (1987) A comparison of eukaryotic viral 5'-leader sequences as enhancers of mRNA expression in vivo. *Nucleic Acids Res.* 15, 8693-8710.
- Genetic Technology News (1990) (June) pp. 8 and 11.
- Gooch, C.F. and Monica, T. (1990) Environmental effects on protein glycosylation. *Bio/Technology* 8, 421-426.
- Gordon, K., Lee, E., Vitale, J.A., Smith, A.E., Westphal, H. and Hennighausen, L. (1987) Production of human tissue plasminogen activator in transgenic mouse milk. *Bio/Technology* 5, 1183-1187.
- Gorodetsky, S.I., Tkach, T.M. and Kapelinskaya, T.V. (1988) Isolation and characterization of the *Bos taurus* beta-casein gene. *Gene* 66, 87-96.
- Grabowski, H., Le Bars, D., Chêne, N., Attal, J., Malienou-N'Gassa, R., Puissant, C. and Houdebine, L.M. (1991) Measurements of rabbit whey acid protein concentration in milk, serum, mammary gland extract and culture medium using a specific radioimmunoassay. A comparison with rabbit alpha- and beta-casein gene concentration. *J. Dairy Sci.* 74, 4143-4154.
- Greenberg, N.M., Anderson, J.W., Hsueh, A.J.W., Nishimori, K., Reeves, J.J., De Avila, D.M., Ward, D.N. and Rosen, J.M. (1991) Expression of biologically active heterodimeric bovine follicle-stimulating hormone in milk of transgenic mice. *Proc. Natl. Acad. Sci. USA* 88, 8327-8331.
- Groenen, M.A.M., Dijkhof, R.J.M., Van der Poel, J.J., Van Diggelen, R. and Verstege, E. (1992) Multiple octamer binding sites in the promoter region of the bovine alpha2-casein gene. *Nucleic Acids Res.* 20, 4311-4318.
- Günzburg, W.H., Salmons, B., Zimmermann, B., Müller, M., Erfle, V. and Brem, G. (1991) A mammary-specific promoter directs expression of growth hormone not only to the mammary gland, but also to bergman glia cells in transgenic mice. *Mol. Endocrinol.* 5, 1123-1133.
- Hall, L., Emery, D.C., Davies, M.S., Parker, D. and Craig, R.K. (1987) Organization and sequence of the human alpha-lactalbumin gene. *Biochem. J.* 242, 735-742.
- Halter, R., Carnwath, J., Espanion, G., Herrmann, D., Lemme, E., Niemann, H. and Paul, D. (1993) Strategies to express factor VIII gene constructs in the ovine mammary gland. *Theriogenology* 39, 137-149.
- Hamamoto, H., Sugiyama, Y., Nakagawa, N., Hashida, E., Matsunaga, Y., Takemoto, S., Watanabe, Y. and Okada, Y. (1993) A new tobacco mosaic virus vector and its use for the systemic production of angiotensin-I-converting enzyme inhibitor in transgenic tobacco and tomato. *Bio/Technology* 11, 930-932.
- Harris, S., McClenaghan, M., Simons, J.P., Ali, S. and Clark, J.



- A.J. (1990) Gene expression in the mammary gland. *J. Reprod. Fert.* 88, 707–715.
- Attiatt, A. (1990) Antibodies produced in plants. *Nature* 344, 469–470.
- Hodgson, J. (1993) Expression systems: A user's guide. Emphasis has shifted from the vector construct to the host organism. *Bio/Technology* 11, 887–893.
- Huang, M.T.F. and Gorman, C.M. (1990) The simian virus 40 small-t intron, present in many common expression vectors, leads to aberrant splicing. *Mol. Cell. Biol.* 10, 1805–1810.
- Ivankevich, S., Kolman, J.L., Bodnar, J.W. and Miller, G. (1992) A nuclear matrix attachment region organizes the Epstein-Barr viral plasmid in Raji cells into a single DNA domain. *EMBO J.* 11, 1165–1176.
- Olivet, G., Devinoy, E., Fontaine, M.L. and Houdebine, L.M. (1992a) Structure of the gene encoding rabbit alpha(s1)-casein. *Gene* 113, 257–262.
- Olivet, G., Devinoy, E., Fontaine, M.L. and Houdebine, L.M. (1992b) Structure of rabbit alpha(s1)-casein gene. *Gene* 11, 257–262.
- Olson, W.K., Yu Lee, L.Y., Clift, S.M., Brown, T.L. and Rosen, J.M. (1985) The rat casein multigene family. Fine structure and evolution of the beta casein gene. *J. Biol. Chem.* 260, 7042–7050.
- Orlowski, R. and Schedl, P. (1991) A position-effect assay for boundaries of higher order chromosomal domains. *Cell* 18, 941–950.
- Orlowski, D. and Wall, R.J. (1988) Identification of specific gene sequences in preimplantation embryos by genomic amplification: detection of a transgene. *Mol. Reprod. Dev.* 1, 57–62.
- Clausner, R.D. and Harford, J.B. (1989) Cis-trans models for post-transcriptional gene regulation. *Science* 246, 870–872.
- Łoczan, D., Hobom, G. and Seyfert, H.M. (1991) Genomic organization of the bovine alpha-S1 casein gene. *Nucleic Acids Res.* 19, 5591–5596.
- Łozak, M. (1991) An analysis of vertebrate mRNA sequences: intimations of translational control. *J. Cell Biol.* 115, 887–903.
- Crimpenfort, P., Rademakers, A., Eyestone, W., Van Der Schans, A., Van Der Broek, S., Kooiman, P., Kootwijk, E., Platenburg, G., Pieper, F., Strijker, R. and De Boer, H. (1991) Generation of transgenic dairy cattle using in vitro embryo production. *Bio/Technology* 9, 844–847.
- Lebas, F. (1970) Description d'une machine à traire les lapines. *Ann. Zootech.* 19, 223–228.
- Lee, K., De Mayo, F.J., Atiee, S.H. and Rosen, J.M. (1988) Tissue-specific expression of the rat beta-casein gene in transgenic mice. *Nucleic Acids Res.* 16, 31027–31041.
- Lee, C.S. and Oka, T. (1992) A pregnancy-specific mammary nuclear factor involved in the repression of the mouse beta-casein gene transcription by progesterone. *J. Biol. Chem.* 267, 5797–5801.
- Levy-Wilson, B. and Fortier, C. (1989) The limits of the DNase I-sensitive domain of the human apolipoprotein B gene coincide with the locations of chromosomal anchorage loops and define the 5' and 3' boundaries of the gene. *J. Biol. Chem.* 264, 21196–21204.
- Maeda, S., Kawai, T., Obinata, M., Fujiwara, H., Horiuchi, T., Saeki, Y., Sato, Y. and Furusawa, M. (1985) Production of human alpha-interferon in silkworm using baculovirus vector. *Nature* 315, 592–594.
- Mason, H.S., Lam, D.M.K. and Arntzen, C.J. (1992) Expression of hepatitis B surface antigen in transgenic plants. *Proc. Natl. Acad. Sci. USA* 89, 11745–11749.
- Massoud, M., Bischoff, R., Dalemans, W., Pointu, H., Attal, J., Schultz, H., Clesse, D., Stinnakre, M.G., Pavirani, A. and Houdebine, L.M. (1991) Expression of active recombinant human alpha1-antitrypsin in transgenic rabbits. *J. Biotech.* 18, 193–204.
- McClenaghan, M., Archibald, A.L., Harris, S., Simons, J.P., Whitelaw, C.B.A., Wilmut, I. and Clark, A.J. (1991) Production of human alpha1-antitrypsin in the milk of transgenic sheep and mice targeting expression of cDNA sequences to the mammary gland. *Anim. Biotechnol.* 2, 2161–2176.
- McKnight, R.A., Shamay, A., Sankaran, L., Wall, R.J. and Henninghausen, L. (1992) Matrix-attachment regions can impart position-independent regulation of a tissue-specific gene in transgenic mice. *Proc. Natl. Acad. Sci. USA* 89, 6943–6947.
- McLaren, A. (1992) The quest for immortality. *Nature* 359, 482–483.
- Meade, H., Gates, L., Lacy, E. and Lonberg, N. (1990) Bovine alpha(s1)-casein gene sequences direct high level expression of active human urokinase in mouse milk. *Bio/Technology* 8, 443–446.
- Meulien, P., Faure, T., Mischler, F., Harrer, H., Ulrich, P., Bouderbala, B., Dott, K., Sainte Marie, M., Mazurier, C., Wiesel, M.L., Van de Pol, H., Cazenave, J.P., Courtney, M. and Pavirani, A. (1988) A new recombinant procoagulant protein derived from the cDNA encoding human factor VIII. *Prot. Eng.* 2, 301–306.
- Mikkelsen, T.R., Chapman, B., Din, N., Ingerslev, J., Kristensen, P., Poulsen, K. and Hjorth, J.P. (1992) Expression of a cytomegalovirus IE-1-factor VIII cDNA hybrid gene in transgenic mice. *Transgenic Res.* 1, 164–169.
- Ninomiya, T., Hoshi, M., Mizuno, A., Nagao, M. and Yuki, A. (1989) Selection of mouse preimplantation embryos carrying exogenous DNA by polymerase chain reaction. *Mol. Reprod. Dev.* 1, 242–248.
- Niwa, M. and Berget, S.M. (1991) Mutation of the AAUAAA polyadenylation signal depresses in vitro splicing of proximal but not distal introns. *Genes Dev.* 5, 2086–2095.
- Ohno, M., Sakamoto, H. and Shimura, Y. (1987) Preferential excision of the 5' proximal intron from mRNA precursors with two introns as mediated by the cap structure. *Proc. Natl. Acad. Sci. USA* 84, 5187–5191.
- Palmiter, R.D., Norstedt, G., Gelinis, R.E., Hammer, R.E. and Brinster, R.L. (1983) Metallothionein-human GH fusion genes stimulate growth of mice. *Science* 222, 809–814.
- Palmiter, R.D., Sandgren, E.P., Avarbock, M.R., Allen, D.D. and Brinster, R.L. (1991) Heterologous introns can en-

- hance expression of transgenes in mice. *Proc. Natl. Acad. Sci. USA* 88, 478–482.
- Palmiter, R.D., Sandgren, E.P., Koeller, D.M. and Brinster, R.L. (1993) Distal regulatory elements from the mouse metallothionein locus stimulate gene expression in transgenic mice. *Mol. Cell. Biol.* 13, 5266–5275.
- Persuy, M.A., Stinnakre, M.G., Printz, C., Mahe, M.F. and Mercier, J.C. (1992) High expression of the caprine beta-casein gene in transgenic mice. *Eur. J. Biochem.* 205, 887–893.
- Pittius, C.W., Hennighausen, L., Lee, E., Westphal, H., Nicols, E., Vitale, J.A. and Gordon, K. (1988) A milk protein gene promoter directs the expression of human tissue plasminogen activator cDNA to the mammary gland in transgenic mice. *Proc. Natl. Acad. Sci. USA* 85, 5874–5878.
- Pursel, V.G., Pinkert, C.A., Miller, K.F., Bolt, D.J., Campbell, R.G., Palmiter, R.D., Brinster, R.L. and Hammer, R.E. (1989) Genetic engineering of livestock. *Science* 244, 1281–1288.
- Qasba, P.K. and Safaya, S.K. (1984) Similarity of the nucleotide sequences of rat alpha lactalbumin and chicken lysozyme genes. *Nature* 308, 377–380.
- Rancourt, D.E., Peters, I.D., Walker, V.K. and Davies, P.L. (1990) Wolffish antifreeze protein from transgenic *Drosophila*. *Bio/Technology* 8, 453–457.
- Reddy, V.B., Vitale, J.A., Wei, C., Montaya-Zavala, M., Stice, S.L., Balise, J. and Robl, J.M. (1991) Expression of human growth hormone in the milk of transgenic mice. *Anim. Biotechnol.* 2, 115–129.
- Riego, E., Limonta, J., Aguilar, A., Pérez, A., de Armas, R., Solano, R., Ramos, B., Castro, F.O. and de la Fuente, J. (1993) Production of transgenic mice and rabbits that carry and express the human tissue plasminogen activator cDNA under the control of a bovine alpha S1 casein promoter. *Theriogenology* 39, 1173–1185.
- Robberson, B.L., Cote, G.J. and Berget, S.M. (1990) Exon definition may facilitate splice site selection in RNAs with multiple exons. *Mol. Cell. Biol.* 10, 84–94.
- Roberts, B., DiTullio, P., Vitale, J., Hehir, K. and Gordon, K. (1992) Cloning of the goat beta-casein-encoding gene and expression in transgenic mice. *Gene* 121, 255–262.
- Schellander, K. and Péli, J. (1992) Mammary specific secretion of human beta-interferon in transgenic mice. *Theriogenology* 37, 291.
- Schmidhauser, C., Caspersen, G.F., Myers, C.A., Sanzo, K.T., Bolten, S. and Bissell, M.J. (1992) A novel transcriptional enhancer is involved in the prolactin and extracellular matrix-dependent regulation of beta-casein gene expression. *Mol. Biol. Cell* 3, 699–709.
- Schöffl, F., Schröder, G., Kliem, M. and Rieping, M. (1993) An SAR sequence containing 395 bp DNA fragment mediates enhanced, gene-dosage-correlated expression of a chimaeric heat shock gene in transgenic tobacco plants. *Transgenic Res.* 2, 93–100.
- Semenza, G.L. and Wang, G.L. (1992) A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Mol. Cell. Biol.* 12, 5447–5454.
- Shamay, A., Pursel, V.G., Wilkinson, E., Wall, R.J. and Hennighausen, L. (1992a) Expression of the whey acidic protein in transgenic pigs impairs mammary development. *Transgenic Res.* 1, 124–132.
- Shamay, A., Pursel, V.G., Wall, R.J. and Hennighausen, L. (1992b) Induction of lactogenesis in transgenic virgin pigs: evidence for gene and integration site-specific hormonal regulation. *Mol. Endocrinol.* 6, 191–197.
- Shamay, A., Pursel, V.G., McKnight, R.A., Alexander, L., Beattie, C., Hennighausen, L. and Wall, R.J. (1991) Production of the mouse whey acidic protein in transgenic pigs during lactation. *J. Anim. Sci.* 69, 4552–4562.
- Shani, M., Barash, I., Nathan, M., Ricca, G., Searfoss, G.H., Dekel, I., Faerman, A., Givol, D. and Hurwitz, D.R. (1992) Expression of human serum albumin in the milk of transgenic mice. *Transgenic Res.* 1, 195–208.
- Shuman, R.M. (1991) Production of transgenic birds. *Experientia* 47, 897–905.
- Sijmons, P.C., Dekker, B.M.M., Schrammeijer, B., Verwoerd, T.C., Van den Elzen, P.J.M. and Hoekema, A. (1990) Production of correctly processed human serum albumin in transgenic plants. *Bio/Technology* 8, 217–221.
- Simons, J.P., McClenaghan, M. and Clark, A.J. (1987) Alteration of the quality of milk by expression of sheep beta-lactoglobulin in transgenic mouse. *Nature* 328, 530–532.
- Simons, J.P., Clark, A.J., Archibald, A.L., Bishop, J.O. and Lathe, R. (1988) Gene transfer into sheep. *Bio/Technology* 6, 179–184.
- Soulier, S., Vilotte, J.L., Stinnakre, M.G. and Mercier, J.C. (1992) Expression analysis of ruminant alpha-lactalbumin in transgenic mice developmental regulation and general location of important *cis*-regulatory elements. *FEBS Lett.* 297, 213–218.
- Stamatoyannopoulos, G. (1991) Human hemoglobin switching. *Science* 252, 383.
- Stinnakre, M.G., Vilotte, J.L., Soulier, S., L'Haridon, R., Charlier, M., Gaye, P. and Mercier, J.C. (1991) The bovine  $\alpha$ -lactalbumin promoter directs expression of bovine trophoblast interferon in the mammary gland of transgenic mice. *FEBS Lett.* 1, 19–22.
- Stinnakre, M.-G., Devinoy, E., Thépot, D., Chêne, N., Bayat-Samardi, M., Grabowski, H. and Houdebine, L.-M. (1992) Quantitative collection of milk and active recombinant proteins from mammary glands of transgenic mice. *Anim. Biotechnol.* 3, 245–255.
- Swanson, M.E., Martin, M.J., O'Donnell, J.K., Hoover, K., Lago, W., Huntress, V., Parsons, C.T., Pinkert, C.A., Pilder, S. and Logan, J.S. (1992) Production of functional human hemoglobin in transgenic swine. *Bio/Technology* 10, 557–559.
- Technology Management Group (1990) Markets for transgenic animals – a worldwide study of applications in breed-stock, production of therapeutic proteins and research animals. *Genetic Technol. News*, pp. 8–11.
- Thépot, D., Devinoy, E., Fontaine, M.L., Hubert, C. and

- Houdébine, L.M. (1990) Complete sequence of rabbit whey acidic protein gene. *Nucleic Acids Res.* 18, 36–41.
- Thépot, D., Devinoy, E., Fontaine, M.L. and Houdebine, L.M. (1991) Structure of the gene encoding rabbit beta-casein. *Gene* 97, 301–306.
- Threadgill, D.W. and Womack, J.E. (1990) Genomic analysis of the major bovine milk protein genes. *Nucleic Acids Res.* 18, 6935–6942.
- Tojo, H., Tanaka, S., Matsuzawa, A., Takahashi, M. and Tachi, C. (1993) Production and characterization of transgenic mice expressing a hGH fusion gene driven by the promoter of mouse whey acidic protein (mWAP) putatively specific to mammary gland. *J. Reprod. Dev.* 39, 145–155.
- Tomasetto, C., Wolf, C., Rio, M.C., Mehtali, M., LeMeur, M., Gerlinger, P., Chambon, P. and Lathe, R. (1989) Breast cancer protein PS2 synthesis in mammary gland of transgenic mice and secretion into milk. *Mol. Endocrinol.* 3, 101579–101584.
- Van Brunt, J. (1990) Transgenics primed for research. *Bio/Technology* 8, 725–728.
- Velander, W.H., Johnson, J.L., Page, R.L., Russell, C.G., Subramanian, A., Wilkins, T.D., Gwazdauskas, F.C., Pittius, C. and Drohan, W.N. (1992) High-level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human protein C. *Proc. Natl. Acad. Sci. USA* 89, 12003–12007.
- Vilotte, J.L., Soulier, S., Stinnakre, M.G., Massoud, M. and Mercier, J.C. (1989) Efficient tissue-specific expression of bovine alpha-lactalbumin in transgenic mice. *Eur. J. Biochem.* 186, 43–48.
- Vilotte, J.L., Soulier, S. and Mercier, J.C. (1992) Sequence of the murine alpha-lactalbumin-encoding cDNA: interspecies comparison of the coding frame and deduced preprotein. *Gene* 112, 251–255.
- Von Kries, J.P., Phi-Van, L., Diekmann, S. and Strätling, W.H. (1990) A non-curved chicken lysosyme 5' matrix attachment site is 3' followed by a strongly curved DNA sequence. *Nucleic Acids Res.* 18, 3881–3885.
- Wall, R.J., Pursel, V.G., Shamay, A., McKnight, R.A., Pittius, C.W. and Hennig-Hausen, L. (1991) High-level synthesis of a heterologous milk protein in the mammary glands of transgenic swine. *Proc. Natl. Acad. Sci. USA* 88, 1696–1700.
- Watakabe, A., Tanaka, K. and Shimura, Y. (1993) The role of exon sequences in splice site selection. *Genes Dev.* 7, 407–418.
- Whitelaw, C.B., Harris, S., McClenaghagh, M. and Simons, J.P. (1992) Position-independent expression of the ovine beta-lactoglobulin gene in transgenic mice. *Biochem. J.* 286, 31–39.
- Wilkins, R.J. and Kuys, Y.M. (1992) Rapid beta-lactoglobulin genotyping of cattle using the polymerase chain reaction animal. *Genetics* 23, 175–178.
- Wilkins, T.D. and Velander, W. (1992) Isolation of recombinant proteins from milk. *J. Cell. Biochem.* 49, 333–338.
- Wright, G., Carver, A., Cottom, D., Reeves, D., Scott, A., Simons, P., Wilmut, I., Garner, I. and Colman, A. (1991) High level expression of active human alpha-1-antitrypsin in the milk of transgenic sheep. *Bio/Technology* 9, 830–834.
- Yang, X. and Anderson, G.B. (1992) Micromanipulation of mammalian embryos: principles, progress and future possibilities. *Theriogenology* 38, 315–335.
- Yom, H.C., Bremel, R.D. and First, N.L. (1993) Mouse mammary tumor virus promoter directs high-level expression of bovine alphaS1 casein in the milk of transgenic heterozygous and homozygous mice. *Anim. Biotechnol.* 4, 89–107.
- Yu S., Deen, K.C., Lee, E., Hennighausen, L., Sweet, R.W., Rosenberg, M. and Westphal, H. (1989) Functional human CD4 protein produced in milk of transgenic mice. *Mol. Biol. Med.* 6, 255–261.
- Zhao, K., Käs, E., Gonzalez, E. and Laemmli, U.K. (1993) SAR-independent mobilization of histone H1 by HMG-I/Y in vitro: HMG-I/Y is enriched in H1-depleted chromatin. *EMBO J.* 12, 3237–3247.

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☒ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☒ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**